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Inactivation of the phospholipase B gene *PLB5* in wild-type *Candida albicans* reduces cell-associated phospholipase A_2 activity and attenuates virulence

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Abstract

Phospholipases are critical for modification and redistribution of lipid substrates, membrane remodeling and microbial virulence. Among the many different classes of phospholipases, fungal phospholipase B (Plb) proteins show the broadest range of substrate specificity and hydrolytic activity, hydrolyzing acyl ester bonds in phospholipids and lysophospholipids and further catalyzing lysophospholipase–transacylase reactions. The genome of the opportunistic fungal pathogen *Candida albicans* encodes a *PLB* multigene family with five putative members; we present the first characterization of this group of potential virulence determinants. Ca*PLB5*, the third member of this multigene family characterized herein is a putative secretory protein with a predicted GPI-anchor attachment site. Real-time RT-PCR gene expression analysis of Ca*PLB5* and the additional Ca*PLB* gene family members revealed that filamentous growth and physiologically relevant environmental conditions are associated with increased *PLB* gene activity. The phenotypes expressed by null mutant and revertant strains of Ca*PLB5* indicate that this lipid hydrolase plays an important role for cell-associated phospholipase A_2 activity and in vivo organ colonization.

Keywords: GPI anchor; Phospholipase; Lysophospholipase; *Candida*; Selection marker; Virulence

Introduction

Phospholipases play a central role in cellular processes such as signal transduction and inflammation through their effect on the metabolism of phospholipids

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and lysophospholipids. Microbial phospholipases further contribute to pathogenesis and virulence through the release or breakdown of bioactive compounds which affect host cell function (Ghannoum, 2000; Schmiel and Miller, 1999; Songer, 1997; Titball, 1993). Substrates for phospholipases are either phospholipids or lysophospholipids, comprised of a polar head group (e.g. ethanolamine, choline, inositol, or serine esterified to phosphoric acid) and one or two non-polar fatty acyl chains esterified to a glycerol backbone. The substrate specificity of phospholipases is determined both by the phospho-headgroups and the chain length and saturation of the fatty acyl side chains. Some phospholipases have broader substrate specificities than others. Phospholipases A_1 and A_2 hydrolyze the ester bonds at the *sn*-1 and *sn*-2 positions of the glycerol moiety, respectively, yielding free fatty acids and 2-acyl or 1-acyl lysophospholipids. The two phosphodiester bonds found in the polar headgroup of the amphipathic phospholipid are cleaved by phospholipase C (first bond), which releases the phospho-head group, and phospholipase D (second bond) which releases only the head group. Phospholipase B (Plb) enzymes possess hydrolytic activities that release both fatty acids from a phospholipid or the remaining fatty acid from a lysophospholipid (lysophospholipase). Additionally, lysophospholipase–transacylase activity is associated with some Plb enzymes allowing these to transfer a free fatty acid to a lysophospholipid and hence produce a phospholipid. Hydrolase and acyltransferase activities have been detected in several fungi including *Saccharomyces cerevisiae*, *Candida albicans*, *Penicillium chrysogenum*, and *Cryptococcus neoformans* (Chen et al., 1997; Lee et al., 1994; Mirbod et al., 1995; Saito et al., 1991; Witt et al., 1984).

C. albicans, the most important fungal opportunistic pathogen (Edmond et al., 1999; Odds, 1988), harbors several phospholipase genes, including genes for phospholipases C and D as well as a gene family with homology to Plb proteins (see Table 1). Although the distinction between phospholipase A, Plb and lysophospholipases is difficult, clear sequence homologs of mammalian or bacterial phospholipase A_1 and A_2 genes seem to be lacking from the genomic repertoire of *C. albicans*. Two Plb genes, Ca*PLB1* and Ca*PLB2,* have been studied in detail (Hoover et al., 1998; Leidich et al., 1998; Mukherjee et al., 2001; Sugiyama et al., 1999); both encode putative secreted proteins with typical signal sequences, but only Ca*PLB1* has been clearly implicated in virulence. Abrogation of CaPlb1 activity by gene inactivation renders the mutant strain-less virulent in experimental animal models (Leidich et al., 1998; Mukherjee et al., 2001). Attack of host cell membranes during tissue invasion and facilitation of adhesion processes through interaction with host-cell phospholipids (Prakobphol et al., 1994, 1997) could be crucial roles for Plb enzymes in pathogenesis of *C. albicans*.

In this study, we report the characterization of a third member of the *PLB* gene family of *C. albicans*, Ca*PLB5*, so designated due to its phylogenetic relationships (see below) with the other phospholipases. Gene expression analysis of the Ca*PLB* gene family using relative quantitations by real-time RT-PCR revealed differential expression profiles with increased Ca*PLB* gene expression under conditions promoting hyphal or pseudohyphal growth. Direct sequencing and sequence analysis of single Ca*PLB5* alleles revealed the presence of two different alleles in several strains of this pathogen. Targeted gene disruption of both alleles in a wild-type strain of *C. albicans* results in reduced phospholipase A_2 (PLA_2) activity in vitro and attenuated virulence as measured by host tissue colonization in a mouse model of systemic infection. Reintroduction of an intact gene copy into the null strain at the original locus results in intermediate levels of organ tissue burden when compared to the wild type and the null mutant.

Materials and methods

Microorganisms, plasmids, and culture media

The *C. albicans* strains used in this work are listed in Table 2. *C. albicans* strain SS is a clinical isolate provided by Dr. Remo Morelli (San Francisco State

Table 1. The *PLB* gene family in the genome sequence of *Candida albicans*

Gene and reference	Haploid assembly 6 ORF(s)	Diploid assembly 19 allele(s)
Ca*PLB1* (Hoover et al., 1998; Leidich et al., 1998)	6.3690	19.689/19.8307
Ca*PLB2* (Sugiyama et al., 1999)	6.1985	19.690/19.8309
Ca*PLB3* (continuous allele, this work)	6.795–6.796	19.1442–19.1443/19.9017–19.9018
Ca*PLB4*	6.6206	19.6594
Ca*PLB5* (this work)	6.4037, 6.6348	19.5102/19.12568

Direct sequencing of the region between the discontinuous ORFs of Ca*PLB3* encoding NH_2- and COOH-terminal portions of the protein revealed a continuous Ca*PLB3* allele (see text). ORF 6.6348 is an NH_2-terminal fragment of ORF 6.4037 (see also www-sequence.stanford.edu/group/candida/index.html, http://www.candidagenome.org/ and references Braun et al., 2005; Jones et al., 2004).

Table 2. Genotypes of *C. albicans* strains used in this study

Strain	Parent and genotype	Source or reference
SS	Clinical isolate	Miyasaki et al. (1994)
SC5314	Wild-type strain	Gillum et al. (1984)
CAI4	SC5314; *ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434*	Fonzi and Irwin (1993)
ATCC 44808	Wild-type strain isolated from human blood	Manning and Mitchell (1980)
KH44	ATCC 44808; $plb5^{LK}$Δ::*FRT-FLP-*MPA^R*-FRT*/$PLB5^{SN}$	This work
KH44-13	KH44; $plb5^{LK}$Δ::*FRT*/$PLB5^{SN}$	This work
KH44-90	KH44-13; $plb5^{LK}$Δ::*FRT*/$plb5^{SN}$Δ::*FRT-FLP-*MPA^R*-FRT*	This work
KH44-91	KH44-90; $plb5^{LK}$Δ::*FRT*/$plb5^{SN}$Δ::*FRT*	This work
KH44-KI	KH44-91; $plb5^{LK}$Δ::*FRT*/$PLB5^{LK}$*-FRT-FLP-*MPA^R*-FRT*	This work
KH44-KL	KH44-KI; $plb5^{LK}$Δ::*FRT*/$PLB5^{LK}$*-FRT*	This work

University, San Francisco, CA, USA). The Ura$^-$ auxotrophic strain *C. albicans* CAI4 was provided by Dr. W. Fonzi (Department of Microbiology and Immunology, Georgetown University Medicine Center, Washington, DC, USA). The wild-type strain *C. albicans* ATCC 44808 was used for gene disruptions (see below). *C. albicans* cells were propagated on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar per liter) or YPD medium at 30 °C. Lee's medium was used to grow *C. albicans* in yeast or filamentous forms (Lee et al., 1975). Host for subcloning and sequencing in pBluescript was *Escherichia coli* DH5α. For screening of the genomic library of *C. albicans* in Lambda FixII (Stratagene, La Jolla, CA, USA) *E. coli* XL1-Blue MRA (P2) was used. Bacteria were grown at 37 °C in Luria–Bertani (LB) medium with suitable supplements.

Cloning and sequencing of Ca*PLB5*

Mapping and sequencing of a 10-kb *Pst*I–*Pst*I genomic DNA fragment, encoding almost the entire ABC transporter gene *MLT1* (Theiss et al., 2002), revealed an incomplete ORF with high similarity to fungal phospholipases. The entire coding region of the putative *PLB* gene Ca*PLB5* was isolated from a *C. albicans* fosmid library (kindly provided by Dr. S. Scherer) using probes derived from the known sequences of the phospholipase and *MLT1*, respectively. Fosmid clone 9d6 was further sequenced by primer walking. Using the oligonucleotide primers AMPLI1 (for oligonucleotide sequences, see Table 3) and AMPLI3 the entire Ca*PLB5* gene with 1 kb 5′-flanking region and 0.4 kb 3′-flanking region was PCR amplified from several *C. albicans* strains and sequenced with the dideoxy sequencing method using a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech) and a LI-COR DNA Sequencer 4000 (Lincoln, NE, USA). The nucleotide sequence of Ca*PLB5* was submitted to GenBank under the accession number AF038128.

Nucleic acid isolation and hybridization

Genomic DNA for cloning and Southern hybridizations was isolated from *C. albicans* as described previously (Millon et al., 1994). Southern hybridizations were carried out with non-radioactive probes using the ECL labeling and detection kit (Amersham Pharmacia Biotech, Freiburg, Germany). A PCR fragment generated with primer pair AMPLI1/PLB1390 was used as probe for Ca*PLB5* hybridizations. Total RNA for Northern hybridizations was isolated with the hot acid phenol method (Ausubel et al., 1989). Membrane-bound RNA was stained with methylene blue before hybridization to check rRNA bands for equal loading. Hybridizations were carried out using standard protocols.

Messenger RNA mapping

The start points of Ca*PLB5* transcription were determined by 5′-RACE (Ausubel et al., 1989). Following reverse transcription with Superscript II (Stratagene) of total RNA with the Ca*PLB5*-specific primer UP-PLIP3, a poly-A tail was added to the first strand by terminal deoxynucleotide transferase action. Subsequently, the 5′-end of the Ca*PLB5* cDNA was amplified with the anchor primer DTNOT and the nested primer ECOMUPL5. PCR fragments were cloned in pBluescript after digestion with NotI and EcoRI, and four clones were sequenced. The polyadenylation sites of Ca*PLB5* mRNA were identified by 3′-RACE: Following the isolation of poly-A$^+$ RNA from total RNA using a Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany) the RNAs were reverse transcribed using primer DTNOT. The 3′-end of the Ca*PLB5* cDNA was amplified with primers RACE3/DTNOT, subsequently cloned and sequenced.

Targeted disruption of the *C. albicans* Ca*PLB5* gene

Sequence-tagged disruption cassettes consisting of the dominant selection marker MPA^R (Köhler et al., 1997,

Table 3. DNA oligonucleotides used in this study

Oligonucleotide	5′-sequence-3′
AMPLI1	TAGTCAAGCTTCGCCATTACAAAGAGC
AMPLI3	CTACTAAGCTTTTCCACTGGTGCATC
AMPLI6	CTACTACTAGTGTCGACGTTTTCCACTGGTGCATC
DTNOT	AATTCGCGGCCGCTTTTTTTTTTTTTTTT
ECOMUPL3	TAGTCGAATTCTTGCTGAGATAG
NOTTAG	TAATTGCGGCCGCTGCAG**NWNNSN**CTCAGTAACAGATACTTG
P3_1340	TATCGTGCCATGCTTAATGG
P3_2305	CCAATCCCGCCATCTATAAC
PLB1390	GACTGCTCGAGTGTAGAACTTGCTGAATCG
RACE3	CAGATGTAAGTGACGAGG
UPPLIP3	TCCACCACTGAATGACAACC
XNTAG	CAGGGACGACTGCTCGAG**NRNNRN**TGTAGAACTTGCTGAATCG
PLB1-A	*GATGAATGGGCAGCATGTGTT*
PLB1-B	*GGCTCACCCTTATAGATGGTACCA*
PLB1-D	*GCATCTCTTACATTGTTCTGTCTGTTC*
PLB1-E (Probe)	*TCTTTCTTGCTCTCTCCGTATGATGGCG*
PLB2-A	*AGGTGAAGACGGTCAGAATGTTC*
PLB2-B	*GCTGATCCATCTGGCCAATT*
PLB2-C	*ATGTTCCCTTGCTTCCATTGA*
PLB2-D	*TGTTTTTATCTGCCGATTGATCA*
PLB2-E (Probe)	*CCACCGTAAGGTAAGCGCAATCTTTGC*
PLB3-A	*AAGAAAGACGTGGTATTGAACAACTG*
PLB3-B	*TGCTTCCATTAGTTAAACTTGAATCAG*
PLB3-C	*TGGTATTGAACAACTGGATCAATGT*
PLB3-E (Probe)	*AAGTACCATCCCAACAATAATT*
PLB4-A	*TCGCTTATGATAATTCGGCTGATA*
PLB4-B	*CCACATCAGGTACATATGGGAAACT*
PLB4-C	*TGCCTCCATGGTAGCATCATAC*
PLB4-E (Probe)	*ATTTGGGAATCAAAGTAATGG*
PLB5-A	*CGCTGCCTCTGTCTCTAGTGTTAG*
PLB5-B	*GATGATCCGGTAGTGGTACTGGTT*
PLB5-C	*CTTGCAGCTAAAACCCACACAA*
PLB5-D	*GATCCGGTAGTGGTACTGGTTTG*
PLB5-E (Probe)	*TGGCGGTACATCTTCCACGACCCA*

Restriction enzyme sites are underlined and sequence tags are in bold. Oligonucleotides for nested and semi-nested real-time PCR are italicized.

2005; Theiss et al., 2002), the inducible site-specific recombinase *caFLP* (Staib et al., 1999; Wirsching et al., 2000) and Ca*PLB5*-specific flanking regions were constructed for gene disruption (see Fig. 4). Initially, a PCR fragment generated with primers AMPLI1 and AMPLI3 from genomic DNA of strain ATCC 44808 was cut with HindIII at the site introduced by AMPLI1 and with BamHI at an internal site in Ca*PLB5*. This fragment was cloned in pSKK (pBluescript SK with the KpnI site removed) to yield pKP1. Removal of the NotI and XhoI sites in pKP1 resulted in pKPNX25. The product of the divergent PCR of pKPNX25 with the primers XNTAG and NOTTAG was cut with XhoI and NotI and subsequently ligated to the XhoI–NotI fragment of pSFI1 (Wirsching et al., 2000) harboring the MPA^R flipper. Following transformation in *E. coli*, bacterial clones carrying plasmids were pooled, their bar-coded plasmids were isolated (pDIS-Pool) and digested with *Apa*I/*Sac*I to yield the linear disruption constructs. Gel-purified DNA fragments were used for transformation of *C. albicans* ATCC 44808 by electroporation as described previously (Köhler et al., 1997). Transformants were plated on SC medium (6.7 g YNB, 0.77 g Complete Supplement Mixture (Bio 101), 20 g glucose, and 15 g agar per liter) containing 10 μg/ml mycophenolic acid (MPA; Sigma-Aldrich). Positive clones were screened for correct integration of the disruption cassette by PCR and Southern hybridization. Excision of the MPA^R flipper necessary for sequential disruption of both Ca*PLB5* alleles was carried out as described previously (Morschhäuser et al., 2005; Wirsching et al., 2000). Amplicons spanning the tagged integration sites of homozygous disruption mutants were generated and sequenced directly. For confirmation of the expected

recombinations on both homologous chromosomes, the four sequence tags flanking the integration sites had to be different.

Reintegration of Ca*PLB5* in a ca*plb5* null mutant

For complementation of the ca*plb5*/ca*plb5* null mutation, an insertion construct with an intact Ca*PLB5* gene copy was generated. For this purpose, a PCR amplicon harboring the Ca*PLB5* gene and its promoter/terminator regions was generated with primers AMPLI1 and AMPLI6 and subsequently digested with SpeI/HindIII. This fragment was ligated to the SpeI–HindIII plasmid backbone of a derivative of pKPNX25 without the SalI site in the multiple cloning site, thereby substituting the insertion cloned in pKPNX25 (see above) with a functional Ca*PLB5* gene plus flanking regions. The resulting plasmid was named p4486. The XhoI–SacII fragment of one disruption plasmid of pDIS-Pool comprised of the MPA^R-flipper and a 3′-fragment of Ca*PLB5* was inserted in p4486 that had been digested with SalI/SacII (the SalI site was introduced by primer AMPLI6), resulting in pPIKOM. The ApaI–SacI fragment of pPIKOM containing the Ca*PLB5*-MPA^R–*Flip*-3′-*Δ*ca*plb5* construct was used for transformation of the null mutant *C. albicans* KH44-91. Transformants were screened by PCR for reintegration of Ca*PLB5* at one of the disrupted loci. One transformant (KH44-KI) was chosen for MPA^R flipper removal and the resulting MPA-sensitive strain KH44-KL was used as the Ca*PLB5* complementation control.

Growth characteristics of ca*plb5* mutants in culture

Growth of mutated strains was compared to wild-type growth in liquid media and agar plates of YPD, SC, RPMI 1640, Lee's media and 10% fetal calf serum at 25, 30 and 37 °C. Growth and filamentation was also tested on Synthetic Low Ammonium Dextrose (SLAD; (Csank et al., 1998)) agar plates following incubation at 37 °C for 7 days. Furthermore, Sabouraud Dextrose Agar, YPD and Lee's pH 6.8 media plates were also supplemented with 10% egg yolk to detect secretion of lipolytic compounds around colonies. Growth in the presence of lysophosphatidylcholine (Lyso-PC; Sigma-Aldrich) or lysophosphatidylinositol (Lyso-PI; Avanti Polar Lipids, Alabaster, AL) was determined by OD_{600} measurement after 24 and 48 h incubation of cultures in 5-ml glass tubes at 30 °C.

Determination of PLA_2 activity

PLA_2 assays were performed as described earlier (Chaitidis et al., 1998). Briefly, 25 μM of 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) was sonicated for 1 min in assay medium containing 10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 5 mM $CaCl_2$. Following preincubation for 5 min, the reaction was started with 50 μg of cell lysate from the wild-type *C. albicans* strain ATCC 44808 or the Ca*PLB5* mutants, which were grown in Sabouraud glucose broth containing 1% peptone medium for 36 h at 37 °C. After a reaction time of 15 min lipids were extracted from the mixture by the Bligh and Dyer method (Bligh and Dyer, 1959). Liberation of arachidonic acid (AA) was measured by reverse-phase HPLC on a Nucleosil-100-7 C_{18} column (Macherey-Nagel, Germany) with a precolumn 5-C_{18}-AB using a solvent system with methanol:water:-acetic acid (85:15:0.01, v/v) at a flow rate of 1 ml/min. Detection was performed with a Diode Array Detector (Shimadzu, Japan). Peaks eluted at about 11.8 min represented AA and were quantitated by peak area using a calibration curve with authentic $cPLA_2$, $sPLA_2$, and $iPLA_2$ standards.

Systemic infection model with Ca*PLB5* mutants

Balb/c mice were infected via the tail vein with 0.4 ml of a 0.9% NaCl solution containing 4×10^5 cells of either the wild-type strain *C. albicans* ATCC 44808, the heterozygous ca*plb5*/Ca*PLB5* mutant KH44-13, the null mutant KH44-91 or the complemented strain KH44-KL. Mice were sacrificed after 3 days post inoculation, and colony-forming units (CFU) of *C. albicans* were determined after homogenization of isolated organs.

Multiplex real-time RT-PCR expression analysis

Strain ATCC 44808 was grown to stationary phase in Lee's medium pH 6.8 at 25 °C, washed two times, subsequently inoculated in fresh Lee's medium pH 4.5 and 6.8, respectively. Cultures were incubated in a rotary shaker at 25 or 37 °C for 3 and 48 h. Total RNA was isolated using glass bead disruption and TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Following DNase I treatment total RNA was further purified with RNeasy (Qiagen). The RT nested or semi-nested real-time PCR was carried out as previously described (Dolganov et al., 2001) with few modifications. A 10-ng aliquot of DNase-treated total *C. albicans* RNA was reverse transcribed using SuperScriptIII First-Strand Synthesis System (Invitrogen) and oligo $(dT)_{20}$ primers. One-tenth of the cDNA was pre-amplified using the Advantage 2 Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA, USA) with a mixture of the respective outer primers A and B (see Table 3). The multiplexed PCR reactions were performed with one cycle at 94 °C for 1 min; 25 cycles at 94 °C for 15 s, 55 °C for 15 s, 70 °C for 15 s; and one cycle at 70 °C for 5 min

in a PTC-200 Peltier Thermal Cycler (MJ Research, South San Francisco, CA, USA). For the real-time PCR reactions, 3 µl of 1:60 diluted pre-amplification material was added to a total 10-µl real-time PCR mix containing QuantiProbe PCR Master Mix (Qiagen, Valencia, CA, USA), a 6-FAM and BHQ-1 labeled probe (primer E), inner primers C and D for Ca*PLB2* and Ca*PLB5* genes or primers C and B for Ca*PLB3* and Ca*PLB4* genes. For Ca*PLB1* the primer combination was A, D, and E. The real-time PCR reactions were performed with one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; and 40 cycles at 95 °C for 15 s and 60 °C for 1 min in an ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All the primers and probes were designed using Primer Express software (Applied Biosystems) and were synthesized by Biosearch Technologies (Novato, CA, USA). The cycle at which the amplification product signal reached a threshold (the threshold cycle, C_T value) was analyzed using SDS2.1 software following the manufacturer's instructions (Applied Biosystems). The threshold was chosen within the geometric (exponential) phase of the amplification curve. To choose a robustly expressed control gene, the C_T values of ~90 genes obtained from cells grown at seven different growth conditions were compared using the GeNorm software (www.wzw.tum.de/gene-quantification/). *EFB1* was stably expressed in this analysis and was used for data normalization. An average C_T value was obtained from the three data sets and normalized to the average C_T value of *EFB1*. For relative quantitation of gene expression, the comparative C_T method was used (Applied Biosystems, 2001) and relative expression was determined as $2^{-\Delta\Delta CT}$.

Results

The Ca*PLB5* gene from *C. albicans*: sequence, annotation and characterization

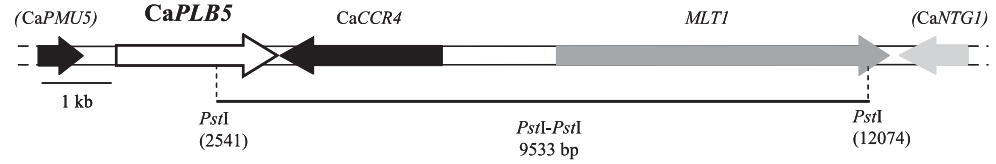
Ca*PLB5*, isolated as described in Materials and methods, lies in close proximity to a 5′-ORF with deduced amino acid sequence similarity to phosphomutases (Ca*PMU5*, allelic orfs 19.5103 and 19.12569 in Assembly 19 of the *C. albicans* genome sequence) and a 3′ ORF 6.4036 (19.5101 and 19.12567) that is located downstream of Ca*PLB5* on the opposite DNA strand encoding a candidal homolog of the *S. cerevisiae CCR4* gene (see Fig. 1A).

Ca*PLB5* shows the highest deduced amino acid sequence identity in databank searches with known fungal type B phospholipases (lysophospholipases); these include *C. albicans* CaPlb1 (identity 46%; Hoover et al., 1998; Leidich et al., 1998; Mukherjee et al., 2001) and CaPlb2 (47%; Sugiyama et al., 1999) and enzymes from *S. cerevisiae* (ScPlb1 46%, ScPlb2 47%, ScPlb3 48% (Lee et al., 1994; Merkel et al., 1999)) or *P. chrysogenum* Plb1 (42%; Masuda et al., 1991). Pfam searches also reveal a lysophospholipase catalytic domain (Accession number: PF01735; from amino acids 177–670; Fig. 1B) within CaPlb5 which is found in other Plb enzymes and cytoplasmic PLA_2.

TBlastn analysis of the *C. albicans* genome Assembly 19 (http://www-sequence.stanford.edu/group/candida/) with the CaPlb5 sequence revealed additional ORFs with a high degree of homology to this protein and to the other fungal Plbs (see Table 1): The protein encoded by orf19.6594 (only one allele in Assembly 19) shares 52% identity and is the most closely related protein to CaPlb5; this gene has been annotated as Ca*PLB4* by the *C. albicans* Genome Annotation Consortium (Braun et al., 2005). ORFs 19.1442/9017 and 19.1443/9018 are adjacent to each other on contigs 10119/20119 of the recent Assembly 19 of the *C. albicans* genome. According to their sequence similarities, they could encode the NH_2-terminal and COOH-terminal portion of a fifth Plb, which we denote CaPlb3 due to its closer phylogenetic relationship with CaPlb1 and CaPlb2 (see below). Since the short region separating these ORFs does not harbor canonical splice sites for an intron, we resequenced the region to check whether Ca*PLB3* is likely to be a pseudogene. A PCR product was generated from genomic DNA of *C. albicans* SC5314 using the primer pair P3_1340-P3_2305 and sequenced directly. The region between the ORFs showed no sequence ambiguities indicative of allelic differences, however, in comparison to Assembly 19 the TAA stop codon of ORFs 19.1442/9017 was replaced by TTA encoding leucine and a cytidine residue at position 24 of

Fig. 1. Gene organization at the Ca*PLB5-MLT1* locus and primary structure of Ca*PLB5*: (A) schematic representation of the Ca*PLB5* locus with the adjacent genes Ca*CCR4* and *MLT1* as well as the partial ORFs Ca*PMU5* and Ca*NTG1*. (B) Nucleotide sequence of one allele of Ca*PLB5* in *C. albicans* ATCC 44808 and its deduced amino acid sequence. The stop codons of the adjacent genes Ca*PMU5* and Ca*CCR4* are boxed in the promoter and terminator region, respectively. Putative TATA elements in the promoter region are underlined. Transcription start sites are marked by an upward arrow, a polyadenylation site in the terminator is marked by a downward arrow. The sequence region deleted in the gene disruption (see Fig. 4) is underlined with a undulate line. Amino acids KR as possible Kex2p processing sites are indicated by deltas (ΔΔ). The putative NH_2-terminal signal peptide and the hydrophobic COOH-terminal with a putative ω cleavage site are double underlined. The NH_2-terminal serine-rich region unique to CaPlb5 is in bold italics. Non-silent allelic differences to the second allele in ATCC 44808 and to both SC5314 alleles are marked by gray shading. Highly conserved active-site regions in the protein sequence are boxed.

A)



B)

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AAACACAGATTGTTGAGTTGGAAGGGTTGCATTTATGGCATGAAAAGAATTGTAAACTCACTGGTAATGATCAGTTTACCTGGGCACCAG   -1054
ATTCGGAATTAACCGAGATTGGATTGGAACAGGCCAAGGAAAACAGAGAATTTTGGAAATCAGAGCTATTGTTAGGAGCCCCCATTCCTA   -964
GTAAATTCCATACTTCGCCATTACAAAGAGCGTGCAGCACATTAGTTATAACTTGGCATGATCTAAAACCAGTAGAGACGAGGCCAATAG   -874
TGTGTGAAAAATTGAGAGAGAAAATTGGGAAAAACCTAAGTGACAAACGTTCTACGAGATCTGAAATTCACGAAAAGTTCGGTGACACTT   -784
ATGGGTTTATAACAGACGGCTTAGAAGAGGAAGATATATATTTTACTGAGACAAGAGAAACCATGCCAGAACAGTCTCTACGCCTAAACG   -694
GATATTTACAAGATTTGTTTGAACAAGACTGCAAATCGGGCAAAGTTGACAAAAAACTGGCAGTTGATAACATTTTCATCAGCACCACGT   -604
CACATTCTGGCACAATTAGATCTGTTATTAATGTGGTCGGTCATAGGAATTTTACAGTGTCCACGGGCGGTATGCTTCCTATAGTAGTTA   -514
AGGCTACCAGACGGAATTAAAAGTTTGACCAAAATCCGGAATGTTTTATATGCAGTACCAACAGGTGTTATCTGATTTACCTGACATATA   -424
TATGAAGAAAAACCATCATATTCTTTTAATACTTTTTATCAATTGCTATATAATTAACAAACCATTCTTACCAGATTAGGTTTCCCGATT   -334
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CATGAAATTTTAGACTAACGTGGGATAGACGAGGAGCCCATCATACACTTGAACTCGTATATATACCAAACCCAATAAACAAATACATTT    -64
                ↑                                                          ↑
CCAGAGAAGATCTTTTTTTTATTGTTTCGTAATAAATCATCTGTTTTCGCTTCTTCTCCCAACAATGAAGGTTAATTTGAAACTAATAATA     26
                                                                        M  K  V  N  L  K  L  I  I      9

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G  S  I  L  I  S  Q  A  Q  A  I  W  P  F  D  S  S  G  S  S  S  S  S  D  S  S  P  S  E  T     39
                                             T
GGCTCATCTGGTGGAACATTTCCTTTTGACTTGTTTGGTTCAGGCTCGTCTCTGACTCAGTCATCATCTGCCCAGGCATCATCAACTAAG    206
G  S  S  G  G  T  F  P  F  D  L  F  G  S  G  S  S  SL T  Q  S  S  S  A  Q  A  S  S  T  K     69

AGTACATCCGATTCAGCAAGTTCTACAGATTCCTCATTGTTCTCTAGTAGCAATTCAGGCAGTTCATGGTACCAAACGTTTTTAGACGGG    296
S  T  S  D  S  A  S  S  T  D  S  S  L  F  S  S  S  N  S  G  S  S  W  Y  Q  T  F  L  D  G     99

GATTCGGGAGATCAAAAGACAGATTATGCTCCATTTAATTTGACATGTCCTTCAAAAAAAACATTTATACGAACAGCATCCGAACTATCT    386
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CAGCAAGAAAAAGACTATATTCACAAGCGTCAGGAAACAACAAATAAAAACTTGATAGACTTTTTGAGTAAACGTGCAAACCTTTCAGAT    476
Q  Q  E  K  D  Y  I  H  K  R  Q  E  T  T  N  K  N  L  I  D  F  L  S  K  R  A  N  L  S  D    159
            Δ  Δ                                                    Δ  Δ
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F  D  A  K  S  F  I  N  D  N  A  P  N  H  N  I  T  I  G  L  S  F  S  G  G  G  G  Y  R  A  M    189
                                                   G
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L  A  G  A  G  Q  I  L  G  L  D  G  R  Y  E  D  A  N  NK H  G  L  G  G  L  L  D  S  S  T    219

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GACTCTGCAGGAATCAAATCCATAACTACTAACGATACCCTCTATTTGTGTGACGGGGGTGAAGATTTACAAAACGTTCCCTTTTATCCA   1466
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L  I  Q  N  E  R  G  V  D  V  I  F  A  F  D  N  S  A  D  T  N  S  S  W  P  N  G  T  S  I    519
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Fig. 1. (*Continued*)

ORFs 19.1443/9018 was deleted leading to a frameshift that renders the respective allelic Ca*PLB3* ORFs continuous. For the following studies we used the deduced amino acid sequence of the corrected Ca*PLB3* gene.

An amino acid alignment of CaPlb5 and other fungal Plbs was carried out with CLUSTALX (Version 1.8) with default parameters and then manually corrected (data not shown). CaPlb5 shares conserved sequences reported to be essential for phospholipase activity (Ghannoum, 2000) with each of these proteins (boxed in Fig. 1B: SGGGY**R**AM, GL**S**GG and **D**GGEDLQN). The arginine, serine and aspartate residues in bold might constitute the active center triad of these enzymes. Due to an NH_2-terminal extension that includes a serine-rich domain (amino acids 25–91) the deduced protein sequence of CaPlb5 is 754 amino acids in length, resulting in a theoretical M_r of 81,373 and considerably larger than most other known fungal Plbs. Among the completed fungal genome sequences a similar NH_2-terminal extension is only found in a putative Plb of the hemiascomycetous yeast *Debaryomyces hansenii* (Dujon et al., 2004). The aligned Plb protein sequences were used to construct a phylogenetic tree with the maximum parsimony algorithm in PAUP-SEARCH of the Wisconsin Package (Version 10.3; Accelrys Inc., San Diego, CA, USA). As shown in Fig. 2, candidal Plbs appear to form two subclusters together with three *D. hansenii* proteins in this phylogenetic tree, one cluster including CaPlb1, CaPlb2, CaPlb3 and a single *D. hansenii* orthologue, the other cluster the enzymes CaPlb4 and CaPlb5, each with a respective *D. hansenii* orthologue. Overall, the phylogenetic tree constructed with fungal Plbs correlates well with trees derived from other phylogenetic markers (e.g. rDNA; Diezmann et al., 2004). Nodes marking the Hemiascomycetes (yeasts), Euascomycetes (Aspergilli, etc.), Archiascomycetes (*Schizosaccharomyces pombe*), and Basidiomycetes (*Cryptococcus* and *Ustilago*) clearly can be found.

Mapping of the Ca*PLB5* transcript provides evidence that the translational start is likely to be the ATG designated as position 1 (Fig. 1B). Start sites of transcription were localized by 5′-RACE to positions −137 and −90, thereby confirming the ATG at position 1 as the first start codon in the Ca*PLB5* mRNA and concomitantly the NH_2-terminal extension of CaPlb5. Putative TATA elements for promoter activity are marked in Fig. 1B. Using 3′-RACE, a polyadenylation site was identified at position 2300, 39 bp from the end of the Ca*PLB5* coding sequence.

A putative signal peptide with a cleavage site at amino acid 19 was detected at the NH_2-terminus of CaPlb5 by SIGNALP (Nielsen et al., 1997). Presence of an NH_2-terminal signal peptide as well as a COOH-terminal stretch of hydrophobic amino acids suggest that CaPlb5 contains a GPI-anchor at the processed COOH-terminus (see Fig. 1B). The asparagine at position 726 was identified as a potential cleavage site ω for the COOH-terminal GPI-attachment signal peptide using the Big-PI Fungal Predictor (Eisenhaber et al., 2004) for modification site prediction. Analysis of the other Plbs in *C. albicans* revealed potential GPI-anchor sites in CaPlb3 and CaPlb4 (data not shown). De Groot et al. (2003) recently used another algorithm to identify fungal GPI proteins and also denoted the three CaPlbs as GPI

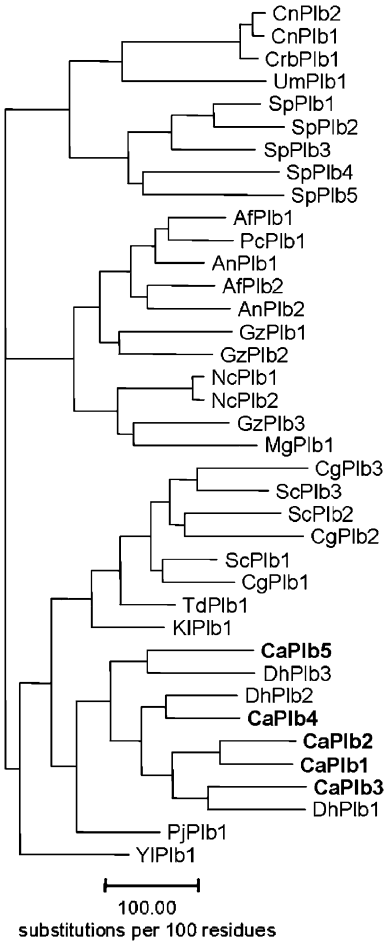

Fig. 2. CaPlb5 and other fungal Plbs. The unrooted phylogenetic tree was generated using the maximum parsimony algorithm in PAUPSEARCH/PAUPDISPLAY of the GCG package with aligned Plb sequences. Organism and Plb (accession number in parentheses): *Aspergillus fumigatus*, AfPlb1 (AAQ85122), AfPlb2 (AAQ85123); *A. nidulans*, AnPlb1 (EAA64795), AnPlb2 (BAD95522); *C. albicans*, CaPlb1 (AAC61890), CaPlb2 (BAA36162), CaPlb3 (orf19.1442/3; the continuous amino acid sequence deduced from direct sequencing of the junction between the adjacent ORFs was used), CaPlb4 (orf19.6594), CaPlb5 (AAF08980); *C. glabrata*, CgPlb1 (AAM16160), CgPlb2 (AAM19335), CgPlb3 (CAG58709); *Cryptococcus bacillisporus*, CrbPlb1 (CAC83081); *Cr. neoformans*, CnPlb1 (AAF65220), CnPlb2 (AAF61964); *Debaryomyces hansenii*, DhPlb1 (CAG88860), DhPlb2 (CAG87754), DhPlb3 (CAG90378); *Gibberella zeae*, GzPlb1 (EAA69595), GzPlb2 (EAA70315), GzPlb3 (EAA73343); *Kluyveromyces lactis*, KlPlb1 (BAA28619); *Magnaporthe grisea*, MgPlb1 (EAA56932); *Neurospora crassa*, NcPlb1 (AAC03052), NcPlb2 (CAE76554); *Penicillium chrysogenum*, PcPlb1 (P39457); *Pichia jadinii*, PjPlb1 (BAC79383); *S. cerevisiae*, ScPlb1 (NP_013721), ScPlb2 (NP_013719), ScPlb3 (NP_014632); *Schizosaccharomyces pombe*, SpPlb1 (NP_593194), SpPlb2 (CAB94277), SpPlb3 (CAB40176), SpPlb4 (CAB57433), SpPlb5 (CAB16354); *Torulaspora delbrueckii*, TdPlb1 (BAA06860); *Ustilago maydis*, UmPlb1 (EAK81777), and *Yarrowia lipolytica*, YlPlb1 (CAG79599). CaPlb family members are depicted in bold.

modified, albeit with differences in the location of the ω sites. Another study only predicted CaPlb5 to be GPI anchored (Lee et al., 2003).

Heterozygosity of Ca*PLB5* alleles

Direct sequencing of PCR products derived from the Ca*PLB5* region in the homozygous strains *C. albicans* CAI4 and ATCC 44808 revealed nucleic acid sequence ambiguities at several positions (for details see Fig. 1B). For corroboration of these sequence variations we sequenced the single intact alleles in heterozygous ca*plb5* mutants. At least two distinct Ca*PLB5* alleles exist in each of the *C. albicans* strains CAI4 and 44808 with sequence variations in the gene coding sequences. Surprisingly, apart from silent mutations (data not shown) the alleles differ even in their amino acid sequence since at two positions in the Ca*PLB5* gene a non-silent C/T transition and a G/T transversion lead to the substitution of leucine to serine (amino acid position 57) and lysine to asparagine (amino acid position 208), respectively. Therefore, we designated one allele as $L_{57}K_{208}$ and the other as $S_{57}N_{208}$. Fig. 1B compares the sequence of the $S_{57}N_{208}$ allele of strain ATCC 44808 with the $L_{57}K_{208}$ allele in the protein sequence. BLAST analysis (Altschul et al., 1990) of the recent Assembly 19 of the *Candida* Genome Sequencing Project (http://www-sequence.stanford.edu/group/candida/search.html) reveals that the same allelic differences in the protein sequences are also present in the Ca*PLB5* alleles (ORFs 19.5102/12568) of the sequenced strain *C. albicans* SC5314 that is the parent strain to CAI4 (Fonzi and Irwin, 1993). Besides a few silent mutations in the nucleic acid sequences (data not shown), there is only one additional amino acid exchange at position 709 between the pairs of allelic sequences of ATCC 44808 and SC5314 (see Fig. 1B). Comparison of the other CaPlb protein sequences in Assembly 19 reveals the presence of further allelic sequence differences in CaPlb1 (D577N; ORFs 19.689/8307) and the NH_2-terminal fragment of CaPlb3 (S346L; ORFs 19.1442/9017).

Ca*PLB5* expression analysis

Ca*PLB5* mRNA expression during growth in YPD medium at 30 °C was monitored from logarithmic to stationary phase by Northern hybridization using total RNAs isolated at various times in the growth cycle (4, 6, 8, 10, 12, 14, 16, and 24 h). The transcript was detected throughout the time course, however, its level increased about three-fold within the 4–10-h period of logarithmic phase and then remained constant from late-log phase to 24 h (data not shown). To address whether Ca*PLB5* mRNA is regulated during the transition from yeast to hyphal cells, we isolated total RNA from *C. albicans*

freshly inoculated from overnight cultures and grown for 3 h in Lee's media (Lee et al., 1975) favoring either growth of yeast forms (pH 4.5, 25 °C) or germ tubes (pH 6.8, 37 °C). Cells were also incubated at intermediate conditions of pH 6.8, 25 °C and pH 4.5, 37 °C. While cells grew in the yeast form at 25 °C, they showed substantial pseudohyphal growth at pH 4.5, 37 °C. The highest levels of Ca*PLB5* mRNA after 3 h incubation as determined by real-time RT-PCR were found in RNA isolated from the germ tubes grown at 37 °C, pH 6.8, while the lowest levels were found in cells grown at 25 °C (see Fig. 3). The other Ca*PLBs* showed the highest expression at pH 4.5, 37 °C, especially Ca*PLB1* with 66-fold induction. After prolonged incubation in the media for 48 h, expression levels for all Ca*PLBs* increased to a large extent, in particular under conditions with low pH (4.5) and elevated temperature (37 °C; see Fig. 3). Overall, Ca*PLB* expression patterns became very similar. Ca*PLB1*, Ca*PLB2* and Ca*PLB3* transcript levels are very low at 3 h in 25 °C, which explains the high induction levels, e.g. at 48 h, 37 °C, pH 4.5. Northern hybridization results (data not shown) corroborate these results since Ca*PLB1*–Ca*PLB3* mRNAs were not detectable in RNA isolated at 3 h, 25 °C.

Generation of Ca*PLB5* mutants

A ca*plb5*/ca*plb5* null mutant of the wild-type strain *C. albicans* ATCC 44808 was generated by sequential targeted gene disruption using the dominant MPA^R resistance gene in conjunction with the inducible *caFLP* recombinase system for marker excision (MPA^R flipper; Wirsching et al., 2000). The heterozygous ca$plb5^{LK}$/Ca$PLB5^{SN}$ mutant *C. albicans* KH44-13 was used in a second round of disruption to inactivate the remaining intact Ca*PLB5* allele finally yielding, after marker excision, the homozygous mutant KH44-91. Fig. 4 shows the disruption strategy and verification by Southern hybridization of genomic DNA. The disruption cassettes included short random sequence tags flanking the FRT sites of the MPA^R flipper construct which could be used to exclude mitotic recombination or chromosome loss as the cause for ca*plb5* homozygosity

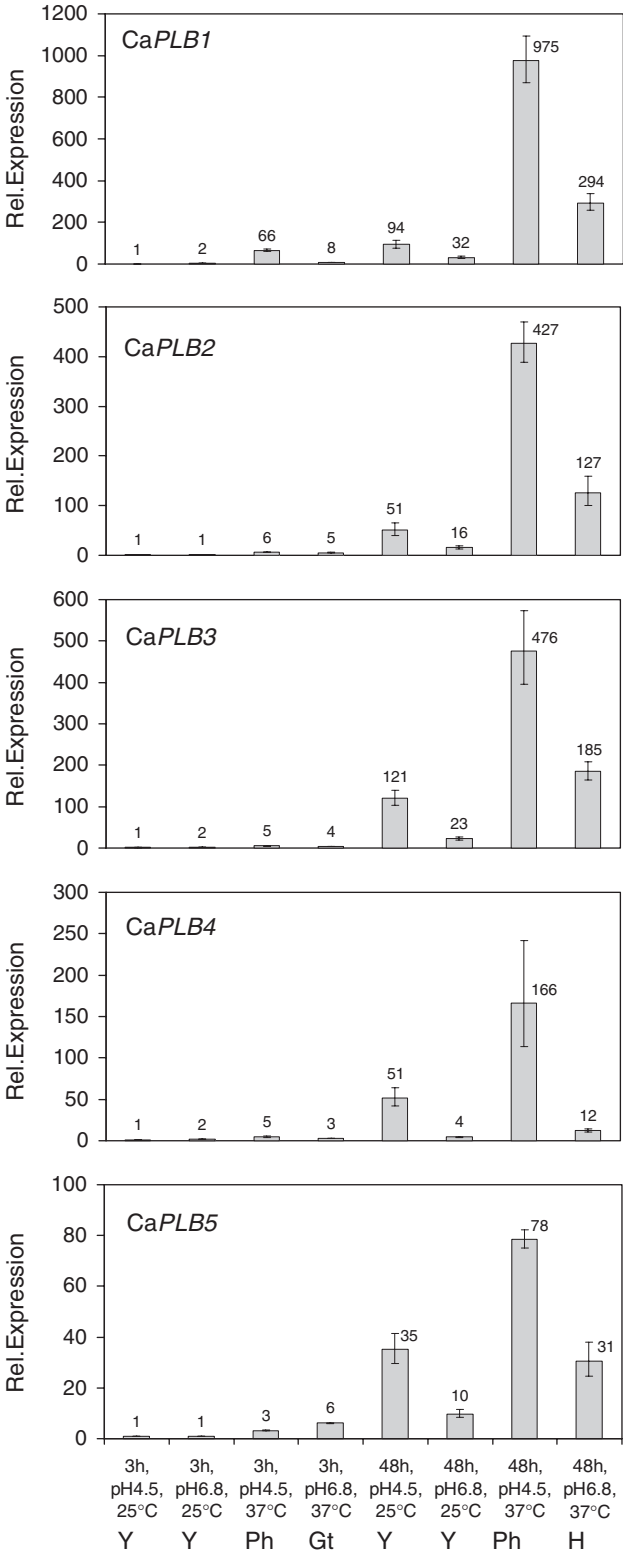

Fig. 3. Relative quantitation of Ca*PLB1*, Ca*PLB2*, Ca*PLB3*, Ca*PLB4* and Ca*PLB5* expression in Lee's medium. *C. albicans* ATCC 44808 cells were grown under the indicated pH and temperatures to promote yeast (pH 4.5 or 6.8 at 25 °C), hyphal growth (pH 6.8, 37 °C) or mixed growth with predominantly pseudohyphae (pH 4.5, 37 °C) and total RNA was isolated after 3 and 48 h of growth. Two-step multiplex real-time RT-PCR was performed to determine the relative expression ($2^{-\Delta\Delta CT}$) of the Ca*PLB* genes. Expression levels of the stably expressed *EFB1* gene were used for normalization as described in the Materials and methods section. Expression levels were calibrated to the respective Ca*PLB* level in Lee's medium pH 4.5 at 25 °C, 3 h. Average values and ranges (error bars) of relative expression in triplicate quantitations are shown. The predominant morphotypes for the respective growth conditions are indicated: yeast (Y), pseudohyphae (Ph), germ tubes (Gt), and hyphae (H).

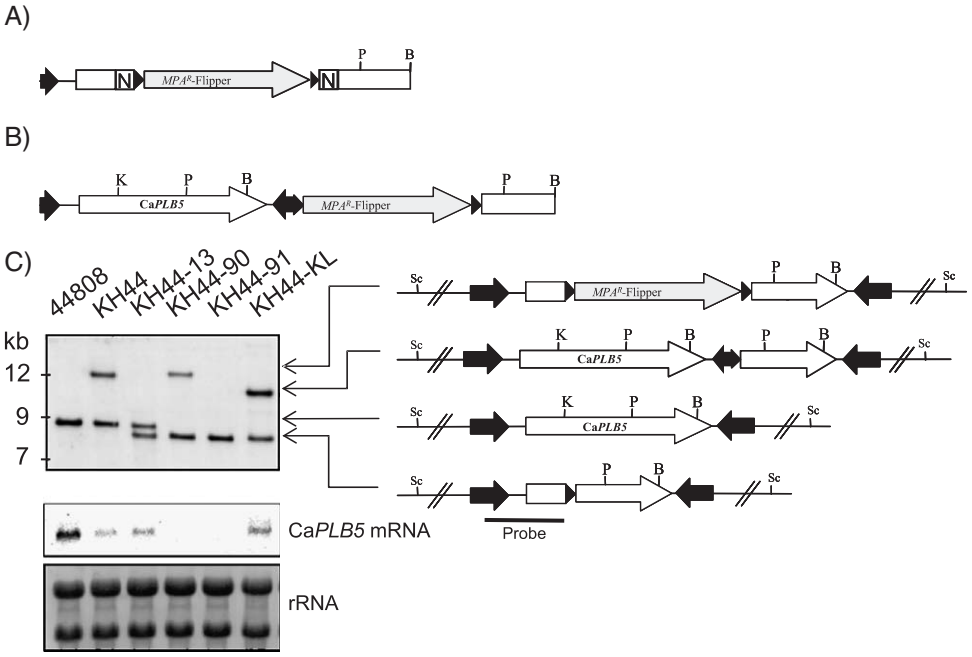

Fig. 4. Ca*PLB5* disruption and reintegration: (A) schematic diagram of a Ca*PLB5* disruption cassette with random sequence tags depicted as "N" (not drawn to scale, tags omitted from subsequent diagrams). Two cassettes with different tags were used sequentially to inactivate the Ca*PLB5* gene. Heterozygosity of the tags present on both alleles could be easily detected by direct sequencing of PCR products across the integration site. Using the MPA^R flipper strategy, each integration step was followed by FLP recombinase-mediated excision of the dominant MPA^R marker leaving only a FRT (shown as black triangle) sequence flanked by tags. (B) Construct used for reintegration of an intact Ca*PLB5* allele in the ca*plb5*/ca*plb5* mutant KH44-91. (C) Southern hybridization analysis of genomic DNA (ScaI digest) isolated from the wild type ATCC 44808, the heterozygous mutants KH44 and KH44-13, the null mutants KH44-90 and KH44-91 as well as strain KH44-KL with a reconstituted Ca*PLB5* allele. Diagrams of the hybridization fragments are shown on the right. Additionally, Northern hybridization results of total RNAs isolated from the aforementioned strains are shown. The null mutants showed no Ca*PLB5* expression, the heterozygous strains and the complemented strain showed reduced levels of expression when compared to the wild type. Methylene blue staining of membrane-bound rRNAs served as loading control. The location of the probe used in Northern and Southern hybridizations is indicated with a black line. B (BamHI), K (KpnI), P (PstI), Sc (ScaI).

in the null mutant KH44-91 (see Materials and methods). The 638-bp deletion in each Ca*PLB5* allele comprised two out of three conserved domains reportedly essential for phospholipase activity (see above and Fig. 1) and resulted in loss of full-length Ca*PLB5* mRNA expression in the null mutants as documented by Northern analysis (Fig. 4). The heterozygous $ca plb5^{LK}$/$CaPLB5^{SN}$ mutants KH44 and KH44-13 showed similarly reduced Ca*PLB5* transcript levels when compared to the wild-type expression. We chose to delete only a portion of the 2262-bp coding sequence because of close proximity of the Ca*CCR4* gene (see Fig. 1).

For verification that the null mutant phenotype was not due to ectopic effects generated during the transformation procedure, a complemented strain with a reintegrated intact $CaPLB5^{LK}$ allele was constructed from KH44-91 using the MPA^R flipper cassette shown in Fig. 4B. Correct integration of the $CaPLB5^{LK}$-MPA^R flipper cassette in strain KH44-KI was verified by PCR, and the subsequent marker excision resulted in strain KH44-KL, a $CaPLB5^{LK}$-*FRT*/$ca plb5^{LK}\Delta$::*FRT* heterozygote that had regained intermediate expression of Ca*PLB5*, similar to the expression level seen in KH44 and KH44-13 (see Fig. 4).

Phenotypic characterization of Ca*PLB5* mutants

Growth of wild-type *C. albicans* ATCC 44808, the ca*plb5* mutants and the reconstituted strain KH44-KL was compared in several media (YPD, Lee's, Spider medium, 10% serum, and synthetic low ammonium dextrose, SLAD; Csank et al., 1998) and no differences in growth rate and morphology were observed. *C. albicans* ATCC 44808 consistently formed the largest precipitation zones around colonies on egg yolk agar (see Materials and methods) of all *C. albicans* strains tested (SC5314, CAI4, SS). This could indicate higher secreted lipolytic activities of ATCC 44808, however, we could detect no differences in precipitation zone

formation between this wild-type strain and its isogenic ca*plb5* mutants on different media with egg yolk. Growth assays with addition of lyso-PC (0.1, 1, 10 mM) and lyso-PI (0.01–0.5 mM), both possible substrates for CaPlb5, revealed in comparison to the wild-type strain no altered susceptibility of the ca*plb5* null mutant to these compounds. However, release of AA from 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC), as measured in a PLA_2 assay of cell lysates, dropped below detection levels in the ca*plb5* null mutant, while the heterozygous mutant and the Ca*PLB5* complemented strain retained approximately half of the activity of the wild type (see Table 4). Thus the Ca*PLB5* alleles encode a significant portion of cell-bound PLA_2 activity in *C. albicans*.

To determine whether, similar to CaPlb1 (Ghannoum, 2000; Ibrahim et al., 1995; Mirbod et al., 1995; Mukherjee et al., 2001), CaPlb5 as a putative phospholipase is involved in virulence, we compared the organ colonization of ca*plb5* mutants to the wild type in a mouse systemic infection model. Disruption of the Ca*PLB5* gene led to an attenuated virulence phenotype of the ca*plb5* null mutant strain KH44-91 of intravenously infected Balb/c mice. Using ANOVA and Dunett error protection CFUs in the liver and the left kidney of strain KH44-91 were identified as significantly reduced when compared to infection with the wild-type strain ATCC 44808, while in the brain as well as in the right kidney CFU reduction was statistically not significant (Fig. 5). The heterozygous mutant KH44-13 with a wild-type Ca$PLB5^{SN}$ allele and the reconstituted strain KH44-KL with a single intact copy of Ca$PLB5^{LK}$ showed intermediate levels of CFU. Homozygous ca*plb5* null mutants generated in the ura-negative strain CAI4 using the *URA3*-blaster technique (Fonzi and Irwin, 1993) showed similar attenuation of organ colonization in vivo (referenced to the *ura3*/*URA3* parent *C. albicans* CAF2-1; data not shown). However, reported difficulties in interpretation of virulence studies that could be encountered with an auxotrophic strain (Chen et al., 2004; Garcia et al., 2001), led us to abandon the *URA3*-blaster strategy and instead use the above described MPA^R flipper method in a wild-type strain.

Table 4. Release of arachidonic acid from 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) by *C. albicans* lysates

Source of enzymatic activity	Arachidonic acid liberation from PAPC (μM/min)
$cPLA_2$ (0.25 IU)	2.4 ± 0.3 (5)
ATCC 44808	1.1 ± 0.2 (3)
KH44-13	0.55 ± 0.1 (3)
KH44-91	<0.01 (3)
KH44-KL	0.73 ± 0.24 (3)

The values of arachidonic acid liberation represent the mean ± SD of the indicated number of experiments (denoted in parentheses). Cytosolic PLA_2 ($cPLA_2$) was used as control. Experimental conditions are described in Materials and methods.

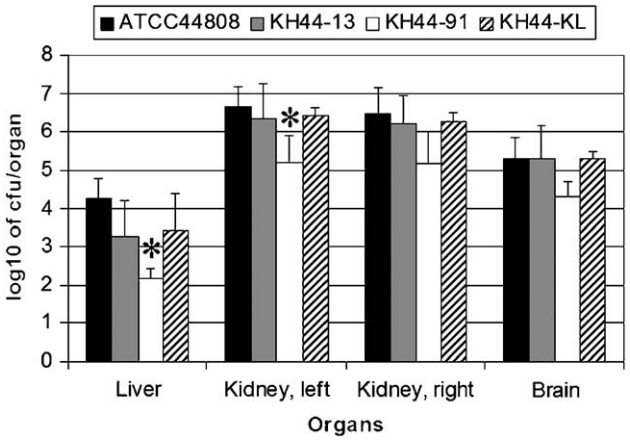

Fig. 5. Organ colonization in a systemic mouse model with the mutant strains in comparison with the wild type. CFUs of infected organs were determined in four mice for each strain. ANOVA and Dunnett error protection was used to calculate statistical significance of differences in organ colonization: CFUs of the ca*plb5* null mutant KH44-91 are significantly reduced compared with wild-type ATCC 44808 CFUs in the liver and the left kidney (marked by an asterisk).

Discussion

Upstream sequences of the *MLT1* gene (Theiss et al., 2002) in *C. albicans* revealed the presence of a third phospholipase gene in this fungus with high amino acid homology to the other known fungal phospholipases B/lysophospholipases. Two additional putative lysophospholipase genes were identified in a TnBLAST survey of the *C. albicans* genome sequence. Therefore, in contrast to three phosholipase B genes in non-pathogenic *S. cerevisiae* and *D. hansenii*, *C. albicans* harbors a gene family of five members encoding these enzymes.

Our real-time PCR analysis of Ca*PLB* expression in Lee's medium revealed that all Ca*PLB* genes are up-regulated in conditions favoring filamentous growth, i.e. pseudohyphae and hyphae formation at elevated temperature (37 °C) and pH 4.5 and 6.8, respectively. Moreover, Ca*PLB* gene expression levels were increased in stationary phase. In fact, using Northern or DNA microarray hybridization Ca*PLB1*, Ca*PLB2* and Ca*PLB3* transcripts were only detectable during later stages of growth (late logarithmic or stationary phases). Interestingly, the highest expression levels of Ca*PLBs* in our study were reached in stationary phase at the physiologically relevant temperature of 37 °C and an acidic pH of 4.5 which is similar to the vaginal pH in

humans. Involvement of gene regulation for filamentous growth in Ca*PLB* gene expression has been shown in recent studies, for example, Ca*PLB1* is derepressed in null mutants of the repressor Ca*TUP1* (Hoover et al., 1998; Kadosh and Johnson, 2005). CaSsn6, another regulator of cell morphology appears to exert control on Ca*PLB3* gene expression since this phospholipase gene is up-regulated in ca*ssn6* mutants (Garcia-Sanchez et al., 2005). The role of other transcription factors in Ca*PLB* gene expression remains to be elucidated. A recent study on the influence of environmental factors like pH and carbohydrate source on in vitro expression of Ca*PLB1* revealed a complex pattern of expression in rich media and chemically defined media supplemented with serum or phospholipids (Mukherjee et al., 2003). Our quantitative RT-PCR study of Ca*PLB1* expression adds to this complexity since we identified a condition in which Ca*PLB1* is in fact strongly induced in chemically defined medium (Lee's medium pH 4.5, 37 °C) independent of supplementation. In vivo expression has been demonstrated for Ca*PLB1* and Ca*PLB2* by RT-PCR in mice (Schofield et al., 2005) and CaPlb1 by immunological detection in host tissue (Mukherjee et al., 2001). We attempted to analyze the in vivo patterns of Ca*PLB5* expression using the In vivo Expression Technology (IVET) adapted to *C. albicans* (Staib et al., 1999), however, the high basal level activity of the Ca*PLB5* promoter led to considerable induction of the FLP recombinase when grown in culture, thus precluding the use of this technology without further adaptation to genes with higher basal expression levels (Bentink and Köhler; unpublished observations).

All the Ca*PLB* genes identified in *C. albicans* are predicted to contain putative signal sequences by SignalP (Nielsen et al., 1997), hence the encoded hydrolytic enzymes are probably secreted under appropriate conditions. The Plbs in *C. albicans* with a hydrophobic COOH-terminus and a high probability for a GPI modification are CaPlb5, CaPlb3 and CaPlb4. Thus, these proteins could be localized to the plasma membrane and/or cell wall, similar to the GPI-modified CnPlb1 protein in *Cr. neoformans* (Djordjevic et al., 2005). Shedding of cell-associated Plbs as suggested for CnPlb1 by glucanase, protease or PI-PLC/PLD activities might also be possible in *C. albicans*.

The serine-rich region following the signal peptide in CaPlb5 and the putative orthologue in *D. hansenii* appears to be absent in phylogenetically more distant fungi. We conducted a preliminary survey of currently available genome sequences of other *Candida* species and found that, e.g. *C. dubliniensis* (http://www.sanger.ac.uk/Projects/C_dubliniensis/) and *C. tropicalis* (http://www.broad.mit.edu/annotation/fungi/candida_tropicalis/) also harbor a CaPlb5 orthologue with an extended NH_2-terminus while more distantly related *C. guillermondi* (http://www.broad.mit.edu/annotation/fungi/candida_guilliermondii/) and *C. lusitaniae* (http://www.broad.mit.edu/annotation/fungi/candida_lusitaniae/) seem to lack it. This stretch of amino acids offers a high density of possible *O*-glycosylation and phosphorylation sites, however, its biological role is unclear. Interestingly, two Kex2 proteinase cleavage sites are located adjacent to the COOH-terminus of this region (position 138 and 153) indicating a potential prepropeptide configuration for processing of CaPlb5. One potential cleavage site is present in the *D. hansenii* orthologue. Newport et al. (2003) recently conducted a survey of putative Kex2 substrates in the *C. albicans* genome and identified several (potentially) secreted hydrolases like secreted aspartyl proteases and sphingomyelinases with Kex2 sites. CaPlb5 probably failed to be recognized by the search algorithm employed in this study, because of too restrictive search parameters. Whether CaPlb5 is indeed processed by the Kex2 proteinase or other processing enzymes remains to be elucidated.

Intrastrain heterozygosity in two amino acid residues of the two alleles present in each of the three *C. albicans* strains tested (ATCC 44808, CAI4 and SC5314) suggests that these exchanges might be of biological importance. CAI4 is derived from SC5314, but ATCC 44808 has a very different CARE2 hybridization pattern of genomic DNA (our unpublished observations) and therefore is likely to be only distantly related to SC5314. We are currently investigating whether the encoded allozymes are functionally different and differentially expressed in vitro or during infection. A survey of further *C. albicans* strains will help to judge the significance of these allelic differences and resolve whether the two amino acid substitutions are compensatory or unrelated. Differential in vivo expression of the two *SAP2* alleles in CAI4 which contain two conserved amino acid exchanges and differ in pentameric repeat structures of their promoter regions, has recently been demonstrated (Staib et al., 2002).

As other similar fungal Plb enzymes, CaPlb5 could be bifunctional with a lysophospholipase–transacylase activity in addition to the hydrolase activity that removes *sn*-1 and *sn*-2 fatty acids from phospholipids and lysophospholipids. Thus, CaPlb5 and other CaPlbs under different growth conditions may account for the phospholipase A activities reported in early studies on *C. albicans* phospholipases (reviewed in Ghannoum, 2000). While its range of biochemical activities and substrates still has to be determined, our findings on AA release from PAPC by a putative PLA_2 activity of CaPlb5 pose an intriguing question about the functional role of this enzyme in pathogenesis. Breakdown of host cell membranes could not only lead to cell lysis, but also to the generation of bioactive lipid mediators derived from host membrane components like AA. Hydrolysis of host phospholipids and release of free fatty acids may

provide precursors for pro- or anti-inflammatory compounds, e.g. eicosanoids such as prostaglandins, leukotrienes and lipoxins that might play an important role in pathogenesis (Noverr et al., 2003; Serhan, 2002).

Secreted phospholipases have been recognized as important virulence factors in microbial infections including candidiasis (Ghannoum, 2000). A Ca*PLB1* null mutant showed attenuated virulence in murine models of hematogenously disseminated candidiasis and oral-intragastric infection (Leidich et al., 1998; Mukherjee et al., 2001). CaPlb5 or the other additional members of the CaPlb family could account for the residual Plb activity and virulence in ca*plb1* mutants. Cell-associated enzymes may be especially important while *Candida* is in close contact with host cells, e.g. during invasion. However, many environmental and cell biological factors (morphology) are likely to determine the temporal and spatial expression and secretion of the different Plbs in vivo. The composition of host cell membranes could influence Plb expression in the fungi; therefore the ca*plb5* mutation might be more detrimental in liver than in other organs, resulting in the most significant attenuation in this organ. Ultimately, further functional characterization and in vivo expression analyses will discern the individual roles of Plb family members in candidal virulence.

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